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Fnr, NarP, and NarL Regulation of *Escherichia coli* K-12 *napF* (Periplasmic Nitrate Reductase) Operon Transcription In Vitro

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The expression of several *Escherichia coli* operons is activated by the Fnr protein during anaerobic growth and is further controlled in response to nitrate and nitrite by the homologous response regulators, NarL and NarP. Among these operons, the *napF* operon, encoding a periplasmic nitrate reductase, has unique features with respect to its Fnr-, NarL-, and NarP-dependent regulation. First, the Fnr-binding site is unusually located compared to the control regions of most other Fnr-activated operons, suggesting different Fnr-RNA polymerase contacts during transcriptional activation. Second, nitrate and nitrite activation is solely dependent on NarP but is antagonized by the NarL protein. In this study, we used DNase I footprint analysis to confirm our previous assignment of the unusual location of the Fnr-binding site in the *napF* control region. In addition, the in vivo effects of Fnr-positive control mutations on *napF* operon expression indicate that the *napF* promoter is atypical with respect to Fnr-mediated activation. The transcriptional regulation of *napF* was successfully reproduced in vitro by using a supercoiled plasmid template and purified Fnr, NarL, and NarP proteins. These in vitro transcription experiments demonstrate that, in the presence of Fnr, the NarP protein causes efficient transcription activation whereas the NarL protein does not. This suggests that Fnr and NarP may act synergistically to activate *napF* operon expression. As observed in vivo, this activation by Fnr and NarP is antagonized by the addition of NarL in vitro.

In *Escherichia coli* complex regulatory mechanisms control the synthesis of anaerobic respiratory enzymes. The master switch (anaerobic induction) is mediated by transcriptional regulator protein Fnr (reviewed in reference 15). In most cases the Fnr protein binds to a site (consensus, TTGAT-N_4-ATCAA) centered approximately 41.5 bp upstream from the transcription start point. This is true for the operons encoding many anaerobic enzymes including nitrate reductase-A (*narGHJI*), fumarate reductase (*frdABCD*), formate dehydrogenase-N (*fdnGHI*), and cytoplasmic (*nirBDC*) and periplasmic (*nrfABCDEFG*) nitrite reductases (8, 15).

The Fnr protein is homologous to the well-characterized transcriptional regulator Crp (cyclic AMP receptor protein). For naturally occurring Crp-activated promoters the Crp-binding site is located at various distances upstream of the promoter. Furthermore, it has been demonstrated that the Crp protein activates the transcription of synthetic promoters when the Crp-binding site is located at various distances upstream of the transcription start site (14). Studies indicate that the location of the Crp-binding site determines the mechanism of transcription activation with respect to interactions between Crp and RNA polymerase (reviewed in reference 3). Consequently, Crp-activated promoters have been divided into different classes determined by the location of the Crp-binding site.

Despite the similarity between Fnr and Crp, early observations that all naturally occurring Fnr-dependent promoters of *E. coli* had the Fnr-binding site close to position −41.5 led to speculation that Fnr was limited to only one mechanism of transcription activation. However, deletion and mutational analyses of the Fnr-activated *napF* (*aeg-46.5*; encoding periplasmic nitrate reductase) operon control region indicated that the Fnr-binding site is at position −64.5. By analogy with Crp-dependent promoters this suggested that it is a naturally occurring example of a second class of Fnr-dependent promoters (9). This possibility is supported by the demonstration that an engineered Fnr protein recognizes the Crp-binding site of the *lac* promoter at position −61.5 to activate *lac* expression (24). Furthermore, Fnr activates the transcription of synthetic promoters with the Fnr-binding site at position −61.5 (or further upstream) and the mechanism of activation is distinct from that when the Fnr-binding site is at position −41.5 (1, 29–31). However, it should be noted that the precise interactions between Fnr and RNA polymerase at the different classes of promoters are not identical to those between Crp and RNA polymerase (2, 29).

Respiratory gene expression is also regulated in response to nitrate and nitrite, the preferred anaerobic electron acceptors. Nitrate and nitrite control is mediated by homologous DNA-binding response regulators (NarL and NarP), which communicate with homologous sensor proteins (NarX and NarQ) (reviewed in references 8 and 26). The NarL and NarP proteins recognize heptamer binding sites that resemble the consensus TACYYMT (where Y = C or T and M = A or C) (6, 12, 20, 27). Both NarL and NarP bind to heptamers organized as inverted repeats with 2-bp spacing. In addition NarL, but not NarP, can also bind to heptamers in other arrangements (10). There are several distinct patterns of operon expression known, including induction by nitrate (e.g., for the *narG* and *fdnG* operons), repression by nitrate (e.g., for the *frdA* oper-

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on), induction by nitrate or nitrite (e.g., for the *nirB* operon), and induction by nitrite and inhibition by nitrate (e.g., for the *nrfA* operon). Despite all of this complexity some generalizations can be made with regard to operon control region architecture. Activation by NarL or NarP occurs when one or both of these proteins bind upstream of an Fnr-binding site centered at −41.5. In other cases, NarL-dependent repression is mediated by the binding of NarL to sites downstream of the Fnr-binding site (reviewed in references 8 and 26). The *napF* operon control region is the one exception to these generalizations about NarL- and NarP-dependent regulation.

napF operon expression is induced by nitrate or nitrite (5, 9, 22). This activation is solely dependent on the NarP protein, unlike all other operons studied, for which activation is dependent on NarL only or on either NarL or NarP (8, 26). The NarP-binding site of the *napF* control region is centered at position −44.5 (downstream of the Fnr-binding site), a more promoter-proximal location than those of other NarP- or NarL-activated promoters. The NarL protein is also able to bind to this −44.5 site but does not activate transcription. However, by competing with NarP for the −44.5 binding site, NarL antagonizes NarP-dependent activation. The inability of NarL to activate *napF* operon expression led us to hypothesize that NarL is deficient in the mechanism by which NarP activates transcription from the −44.5 binding site (9). By contrast, both NarL and NarP are competent to activate transcription activation from the more upstream binding sites of other operon control regions.

In the present study we sought to confirm the location of the Fnr-binding site in the *napF* operon control region and to demonstrate that Fnr may activate transcription by an atypical mechanism. We also reproduced the regulation of the *napF* promoter by Fnr, NarP, and NarL in vitro and confirmed and extended our previous conclusions about *napF* operon regulation.

MATERIALS AND METHODS

Strains and plasmids. For routine manipulations, plasmids were propagated in strain DH5α [φ80d Δ(*lacZ*)M15 Δ(*argF-lac*)*U169 endA1 recA1 hsdR17 deoR thi-1*]. Strain VJS5577 (Table 1) is a derivative of VJS676 [Δ(*argF-lac*)*U169*] with a single-copy λΦ(*napF-lacZ*) operon fusion (includes the *napF* control region from positions −146 to +305 [9]) and *fnr-271*::Tn*10*-, *narL249*::Ω-Sp-, and *narP253*::Tn*10*d(Cm)-null alleles that were introduced by P1 *kc*-mediated transduction. Plasmids containing fnr^+ (and the mutant derivatives) were a gift from Steve Busby (University of Birmingham, Birmingham, United Kingdom) and have been described previously (30).

Culture media and conditions. Defined, complex, and indicator media for routine genetic manipulations were used as described previously (11). When necessary media were routinely supplemented with ampicillin (200 μg/ml), tetracycline (25 μg/ml), chloramphenicol (20 μg/ml), or spectinomycin (30 μg/ml). Cultures for β-galactosidase assay (Table 1) were grown in 3-(*N*-morpholino)-propanesulfonic acid (MOPS)-buffered minimal medium (pH 8.0) with glucose as the sole carbon source (25). This medium was supplemented with tetracycline (25 μg/ml) and ampicillin (60 μg/ml); 10% (vol/vol) Luria-Bertani broth was added to stimulate growth. Culture densities were measured with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) with a no. 66 (red) filter.

β-Galactosidase assay. β-Galactosidase activities were determined at room temperature (approximately 21°C) in permeabilized cells exactly as described previously (19). Activities are expressed in arbitrary units (21). Each culture was assayed in duplicate, and reported values were averaged from three independent cultures, the standard errors of which were not more than 15%.

Protein purification. Fnr (D154A) protein (mutant Fnr protein with an aspartate-to-alanine substitution at position 154) was purified as described previously (18, 32) except that a heparin agarose chromatography step followed the Q-Sepharose anion exchange chromatography to remove a contaminating nuclease activity. The purified Fnr (D154A) protein was 10% active in site-specific DNA binding, as determined previously (32). The MBP-NarL and MBP-NarP proteins were purified exactly as described previously (9). All stated protein concentrations refer to the monomeric concentration.

DNase I footprinting. DNase I footprinting assays of the *napF* control region with Fnr (D154A) were done as described previously (10) except that acetyl phosphate was not included in the reaction mixtures. Reaction mixtures were incubated at 37°C for 30 min to reach equilibrium prior to the addition of DNase I. The *napF* control region fragment was generated from plasmid pVJS1523 by PCR and labeled with ^{32}P on the bottom strand as described previously (9).

Construction of a *napF* control region template plasmid for in vitro transcription assays. The *napF* control region from −202 to +91 (with respect to the transcription start site) was amplified from plasmid pVJS1515 (9) by PCR. The primers were 5′-***CCTGCAAGC***TTAGTGTTAAATTCTAATGAGAGAG-3′ and 5′-***CCGAGGATCC***GCATCAATCTTCACATTGACCTTC-3′. These primers had unannealed tails (boldface and italics) to generate *Hin*dIII and *Bam*HI sites. The product fragment was digested with *Hin*dIII and *Bam*HI and cloned into plasmid pUC19-*spf′* (13) to generate template plasmid pVJS2111. This places the *napF* promoter approximately 200 bp upstream of the *spf* transcription terminator.

In vitro transcription assays. Supercoiled pVJS2111 plasmid DNA was purified with a plasmid purification kit (Qiagen Inc., Chatsworth, Calif.). Phosphorylation of the MBP-NarL and MBP-NarP proteins was essential for activity in the transcription reactions (data not shown). Therefore, for all of the experiments in this study, the MBP-NarL and MBP-NarP proteins were phosphorylated by incubating them in 50 mM Tris-Cl (pH 7.6)–10 mM $MgCl_2$–50 mM acetyl phosphate for 60 min at 37°C. The phosphorylation reaction mixture was then spun through a Sephadex G-25 column to remove acetyl phosphate, which interfered with the transcription reactions. The transcription reaction buffer contained 40 mM Tris-Cl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, and nucleotide triphosphates (0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.05 mM UTP, and 5 μCi of [α-^{32}P]UTP at 3,000 Ci/mmol). A 20 nM concentration of supercoiled pVJS2111 plasmid DNA was preincubated in reaction buffer for 20 min with Fnr (D154A), phosphorylated MBP-NarP, or phosphorylated MBP-NarL proteins in a total volume of 20 μl. Reactions were initiated with the addition of 50 nM *E. coli* RNA polymerase holoenzyme ($E\sigma^{70}$; Epicentre Technologies, Madison, Wis.). After a further 5-min incubation at 37°C the reactions were terminated by the addition of 10 μl of formamide loading dye. Then, 3 to 6 μl of each reaction mixture was loaded onto a 6% (wt/vol) denaturing polyacrylamide gel. Reactions products were separated by electrophoresis, and the gels were dried and exposed to X-ray film for 4 to 16 h.

The gels were quantitatively analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The ratio of the *napF* transcript to the constitutive *ori* transcript from the plasmid vector was used to quantify changes in transcription from the *napF* promoter. When the phosphorylated MBP-NarP and MBP-NarL proteins were spun through a Sephadex G-25 column to remove acetyl phosphate, 20 to 40% of the protein was lost. This variability meant that slightly different concentrations of phosphorylated MBP-NarP or MBP-NarL protein were used in duplicate experiments. Therefore, the data presented in Fig. 3 to 5 are from single experiments. However, the effects of the various regulatory proteins on *napF* transcription in vitro were reproducible in independent experiments.

RESULTS

The Fnr protein binds to the −64.5 site of the *napF* control region. Deletion and mutational analyses had suggested that the Fnr-binding site of the *napF* control region is centered at position −64.5 (Fig. 1) (9), significantly further upstream than the location of that for other Fnr-dependent promoters (approximately −41.5). Therefore, we wished to confirm the location of the Fnr-binding site by DNase I footprint analysis.

Fnr (D154A) was used in these studies as it has properties that make it more amenable to in vitro experimentation (32). This Fnr (D154A) protein was able to activate Φ(*napF-lacZ*) expression in vivo (data not shown). In vitro, the purified Fnr (D154A) protein weakly protected nucleotides from approximately −55 to −80 on the bottom strand of the *napF* control region from DNase I attack (Fig. 2). This region includes the predicted Fnr-binding site centered at position −64.5 (Fig. 1). The binding of Fnr also resulted in sites becoming hypersensitive to DNase I cleavage around positions −47, −62, and −89, possibly indicative of DNA bending. These results confirm the unusual location of the Fnr-binding site in the *napF* control region.

The *napF* operon has an atypical Fnr-dependent promoter. By analogy with Crp-dependent promoters (3) and synthetic Fnr-dependent promoters (29, 30), we hypothesized that the mechanism by which *napF* expression is activated by Fnr is distinct from the mechanism involved in the activation of most

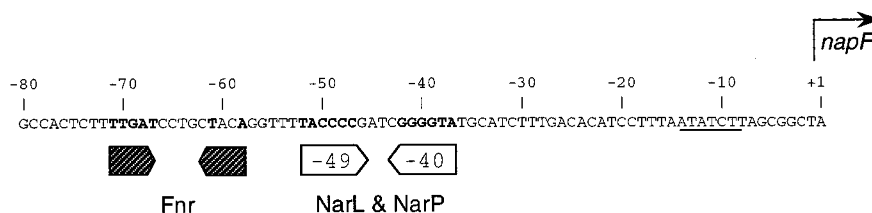


FIG. 1. Architecture of the *napF* operon control region. The in vivo anaerobic transcription initiation site (+1) (5) (data not shown) is indicated by the arrow, and the −10 hexamer is underlined. The Fnr-binding site is indicated by inverted hatched arrows, with residues identical to those of the consensus (TTGAT-N_4-ATCAA) in boldface. The NarP- and NarL-binding site (inverted heptamer sequences) is indicated by numbered arrows. Residues identical to those of the consensus heptamer sequence (5′-TACYYMT-3′) are in boldface. Each heptamer is denoted by the position of the central nucleotide with respect to the transcription initiation site.

other naturally occurring Fnr-dependent promoters. Positive control mutations have identified amino acid side chains of the Fnr protein likely to be involved in transcription activation but not in DNA binding (1, 28–30). One of these substitutions (G85A [Gly at position 85 changed to Ala]) interferes with transcription activation when Fnr is bound at position −41.5 but enhances activation when the Fnr-binding site is at position −61.5 (or further upstream). Another Fnr substitution (S73F) strongly reduces activation by Fnr from −61.5 binding sites (or further upstream) but has a more subtle effect when the Fnr-binding site is at position −41.5 (1, 30). The different effects of these positive-control mutations suggest different mechanisms of Fnr-dependent transcription activation for the two classes of promoter. To determine the class of Fnr-dependent promoters to which the *napF* promoter belongs, we investigated the effect of these Fnr-positive control mutations on Φ(*napF-lacZ*) expression in vivo (Table 1).

To avoid any complications related to binding of the NarL and NarP proteins to the *napF* control region, the effects of the *fnr* mutations were investigated in a *narL narP* double-null derivative of an *fnr*-null strain (VJS5577; see Materials and Methods). When wild-type Fnr was expressed from a multicopy plasmid, Φ(*napF-lacZ*) expression was induced approximately sixfold by anaerobiosis (Table 1). With Fnr (G85A) the anaerobic induction was increased to 15-fold. This increase in transcription activation by Fnr (G85A) is characteristic of an Fnr-dependent promoter with the Fnr-binding site at, or upstream of, position −61.5 (1, 30). With Fnr (S73F), anaerobic activation of Φ(*napF-lacZ*) expression was significantly reduced (Table 1). Once again this is characteristic of an Fnr-dependent promoter with a more upstream Fnr-binding site. Note that the Fnr (G85A) and Fnr (S73F) proteins had the expected effects on expression from synthetic Fnr-dependent control promoters with Fnr-binding sites at positions −41.5 and −71.5 (data not shown) (30).

In vitro transcription from the *napF* promoter. Deletion, mutational, and footprint analyses had indicated that *napF* operon expression in vivo is induced weakly by either Fnr or NarP alone and strongly by Fnr and NarP together. In contrast, the NarL protein antagonizes *napF* operon expression by competing with NarP for a common DNA-binding site (9). We attempted in vitro transcription assays to further investigate this complex pattern of in vivo regulation.

A weak *napF* transcript was detected in reaction mixtures containing only $E\sigma^{70}$ RNA polymerase (Fig. 3). The size of this transcript suggested that the initiation site was in the region identified as the in vivo transcription start site (data not shown) (5). The addition of Fnr (D154A) alone had no significant effect on this basal level of transcription (confirmed by phosphorimager analysis; data not shown), consistent with only very weak activation by Fnr alone in vivo (9). In the presence of either phospho-MBP-NarP or phospho-MBP-NarL fusion proteins alone, there was a slight increase in *napF* transcription (Fig. 3). The most significant induction of the *napF* promoter was observed in the presence of both Fnr (D154A) and MBP-NarP, consistent with in vivo observations (9). However, the amount of *napF* transcript in the presence of Fnr (D154A) and MBP-NarL was similar to that with MBP-NarL only (Fig. 3). It should be noted that NarP and NarL proteins that were separated from MBP by factor X protease cleavage behaved in a

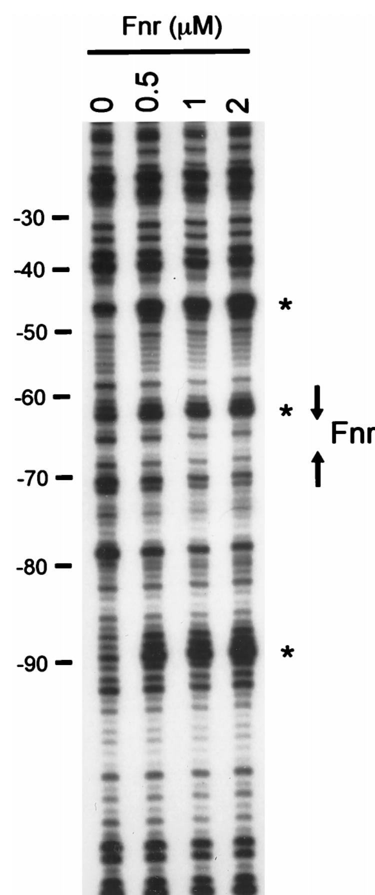


FIG. 2. DNase I footprint analysis of the *napF* operon control region. The control region fragment was labeled on the bottom (template) strand. Each lane is labeled with the concentration of Fnr (D154A) used in the reaction mixture (in micromolar monomers). The number of base pairs from the transcription start site is indicated on the left (a G+A sequencing reaction of the *napF* control region fragment was used as a size marker [not shown]). The location of the Fnr-binding site is indicated by the inverted arrows. Asterisks mark the positions of sites hypersensitive to DNase I cleavage in the reactions with Fnr (D154A).

TABLE 1. Effects of Fnr-positive control mutations on Φ(*napF-lacZ*) expression

pFnr derivative[b]	β-Galactosidase sp act of culture grown[a]:	
	$+O_2$	$-O_2$
Δ*fnr*[c]	16	29
fnr$^+$	17	110
fnr (G85A)	16	250
fnr (S73F)	14	68

[a] Specific activity was determined as described in Materials and Methods and expressed in arbitrary (Miller) units. cultures were aerated ($+O_2$) or were grown anaerobically ($-O_2$).

[b] Strain VJS5577 [*fnr*::Tn*10 narL*::Ω-Sp *narP*::Tn*10*d(Cm) λΦ(*napF-lacZ*)] carrying each of the indicated pFnr plasmid derivatives.

[c] pFnr plasmid derivative with the JK13 deletion (29).

manner identical to that of the intact fusion proteins in all experiments (data not shown).

Interestingly, in the presence of either phospho-MBP-NarP or phospho-MBP-NarL fusion proteins the transcript was slightly shorter than in their absence (Fig. 3). This may indicate that the *napF* transcription start site changes in the presence of MBP-NarP or MBP-NarL.

The results from these initial experiments suggested that Fnr (D154A) and MBP-NarP together activate the *napF* promoter more than either protein does alone. However, it appeared that the same was not true for Fnr (D154A) and MBP-NarL. This possibility was investigated further by quantitative analysis in the next series of experiments.

Both MBP-NarP and Fnr (D154A) are required for maximum *napF* transcription in vitro. To investigate the regulation of *napF* transcription in more detail, we did a series of titration experiments with each of the regulatory proteins. The Fnr (D154A) protein alone (at any concentration) was unable to activate *napF* transcription above the level observed with RNA polymerase alone, but instead caused a slight repression (data not shown). In a control experiment, the activity of the Fnr (D154A) protein was confirmed by its ability to activate transcription of the Fnr-dependent *dmsA* promoter in vitro (data not shown). In the presence of MBP-NarP (7.5 μM), the addition of 0.5 μM Fnr (D154A) increased the amount of *napF* transcript by approximately two- to threefold. Fnr (D154A) was less stimulatory at higher or lower concentrations (data not shown). Therefore, we used 0.5 μM Fnr (D154A) for all of the subsequent reactions.

When increasing amounts of MBP-NarP alone were used in transcription assays, there was a slight increase in *napF* transcription (Fig. 4). In similar titration experiments with MBP-NarL there was also a slight increase in *napF* transcription. The phosphorimager analysis revealed that either MBP-NarP or MBP-NarL alone caused a two- to threefold increase of *napF* transcription above the basal level (Fig. 4). In the presence of both Fnr (D154A) and MBP-NarP there was a sevenfold increase. This compares to the threefold and undetectable increase with MBP-NarP alone and Fnr (D154A) alone, respectively. In contrast, the phosphorimager analysis revealed that in the presence of both Fnr (D154A) and MBP-NarL there was no further increase in *napF* transcription beyond the threefold increase above the basal level observed with MBP-NarL alone (Fig. 4). It is possible that NarP and Fnr may activate *napF* transcription synergistically whereas Fnr and NarL cannot, as discussed below. Note that in a control experiment, the activity of the MBP-NarL protein was confirmed by its ability to activate transcription of the NarL-dependent *fdnG* promoter in vitro (data not shown).

MBP-NarL antagonizes activation of the *napF* promoter by Fnr (D154A) and MBP-NarP in vitro. Mutational and DNase I footprint analysis had indicated that the NarP and NarL proteins are each capable of binding to the −44.5 site of the *napF* control region (Fig. 1) (9). This competitive binding presumably leads to antagonization of NarP-dependent activation by the NarL protein, since only NarP and Fnr together cause maximum *napF* expression. This prediction was tested in vitro by investigating the effect of increasing amounts of MBP-NarL on *napF* transcription in the presence of Fnr (D154A) and MBP-NarP.

In the presence of the optimal concentrations of Fnr (D154A) (0.5 μM) and MBP-NarP (approximately 5 μM) the addition of MBP-NarL decreased the amount of *napF* transcription (Fig. 5). The amount of the *napF* transcript decreased steadily as increasing amounts of MBP-NarL were included in the reaction mixture. With 16 μM MBP-NarL, *napF* transcription was decreased by over 50% (Fig. 5). These results confirm that MBP-NarL decreases MBP-NarP- and Fnr (D154A)-dependent transcription of *napF* in vitro. Since MBP-NarL, either alone or in the presence of only Fnr (D154A), does not decrease (repress) *napF* transcription (Fig. 4), we conclude that MBP-NarL antagonizes MBP-NarP-dependent activation, presumably by binding site competition.

DISCUSSION

The *napF* promoter is one of several regulated by Fnr in response to anaerobiosis and by NarL or NarP in response to nitrate and nitrite. Many of these promoters have similarities in their control region architecture. The Fnr-binding site is typically centered near position −41.5, and activation by nitrate or nitrite is dependent on NarP- and/or NarL-binding sites further upstream (reviewed in reference 8). However, the *napF* control region has a very different architecture, with an Fnr-binding site centered at position −64.5 and activation by NarP being mediated by a binding site downstream, at position −44.5 (Fig. 1). In this study we confirmed the location of the Fnr-binding site at position −64.5 and supported the idea that the mechanism of Fnr-dependent activation of *napF* expression is distinct from that of promoters with the Fnr-binding site at position −41.5. The regulation of the *napF* promoter was also studied in vitro, wherein we reproduced and extended

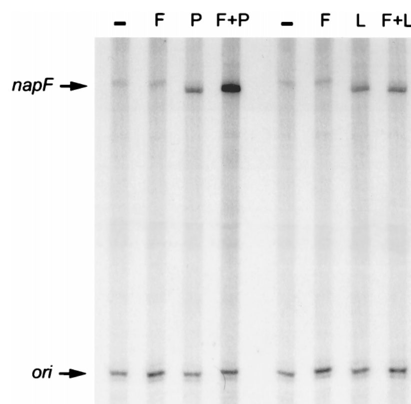
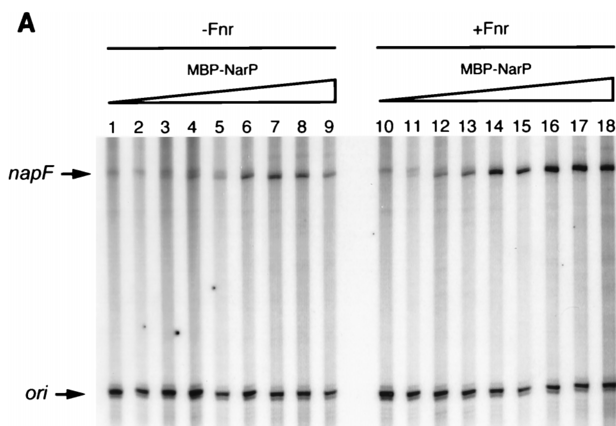
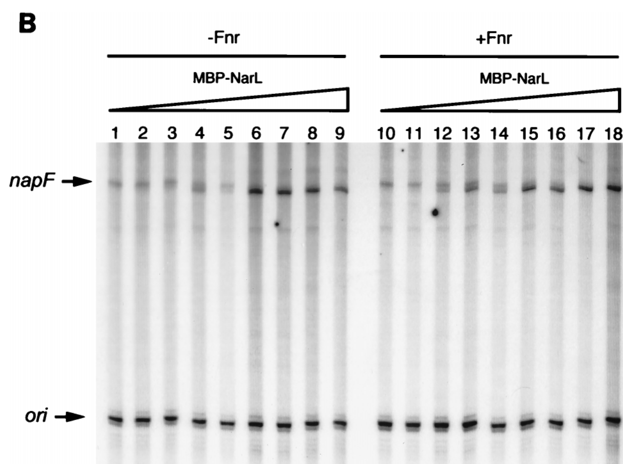


FIG. 3. In vitro transcription from the *napF* promoter. Each multiple-round transcription assay mixture contained 20 nM supercoiled pVJS2111, 50 nM RNA polymerase holoenzyme ($E\sigma^{70}$), and either no further additions (−), 0.5 μM Fnr (D154A) monomers (F), 6 μM phosphorylated MBP-NarP monomers (P), or 12 μM phosphorylated MBP-NarL monomers (L). The *napF* and constitutive plasmid *ori* transcripts are labeled.





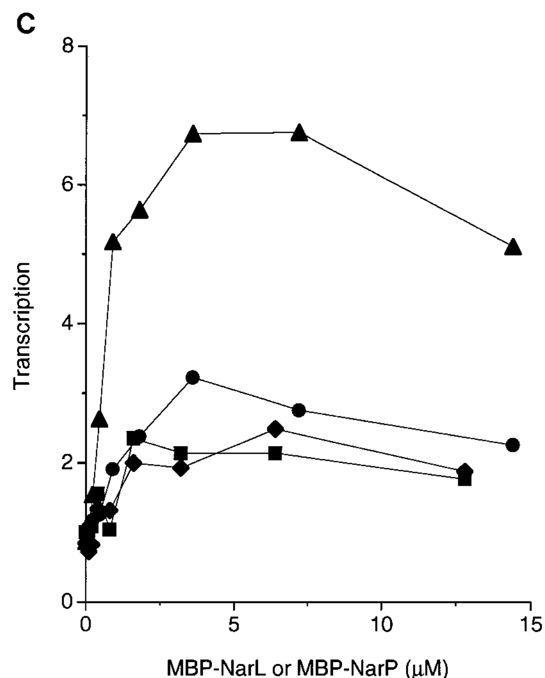


FIG. 4. Both MBP-NarP and Fnr (D154A) are required for maximum *napF* transcription in vitro. (A) Effect of phosphorylated MBP-NarP concentration on *napF* transcription in vitro. Reaction mixtures contained either no Fnr (D154A) (−Fnr) or 0.5 μM Fnr (D154A) monomers (+Fnr). The concentration of phosphorylated MBP-NarP either was zero (lanes 1 and 10) or ranged from approximately 0.11 to 14 μM monomers in twofold increments (lanes 2 to 9 and 11 to 18). (B) Effect of phosphorylated MBP-NarL concentration on *napF* transcription in vitro. Reaction mixtures contained either no Fnr (D154A) (−Fnr) or 0.5 μM Fnr (D154A) monomers (+Fnr). The concentration of phosphorylated MBP-NarL either was zero (lanes 1 and 10) or ranged from 0.1 to 12.8 μM monomers in twofold increments (lanes 2 to 9 and 11 to 18). (C) Representation of the data from the experiments presented in panels A and B, which were analyzed as described in Materials and Methods. For each titration, the amount of *napF* transcript with $E\sigma^{70}$ alone [titrations in the absence of Fnr (D154A)] or with $E\sigma^{70}$ plus Fnr (D154A) [titrations in the presence of Fnr (D154A)] was arbitrarily assigned the value of 1.0. ■, MBP-NarL without Fnr (D154A); ◆, MBP-NarL plus Fnr (D154A); ●, MBP-NarP without Fnr (D154A); ▲, MBP-NarP plus Fnr (D154A).

previous in vivo observations. The results support our hypothesis (9) that the Fnr and NarP proteins act together to cause maximum *napF* operon expression whereas the Fnr and NarL proteins do not.

Location of the Fnr-binding site in the *napF* control region. The homologous Fnr and Crp proteins are each able to activate synthetic promoters with the Crp- or Fnr-binding sites either near position −41.5 or near −61.5 (or further upstream) (14, 30). Activation of these different classes of promoters occurs via different contacts between RNA polymerase and the regulatory protein, although the precise contacts that Fnr and Crp make with RNA polymerase are not identical (2, 29). There are numerous examples of naturally occurring Crp-dependent promoters of each class (e.g., the *lac* and *gal* promoters). However, the *napF* promoter is a rare example of a naturally occurring Fnr-dependent promoter that is in a class distinct from those of the more common promoters with the Fnr-binding site at position −41.5. The fact that most Fnr-dependent promoters have the latter architecture may be due to the fact that many of them are also activated by the NarL protein (e.g., the *narG*, *fdnG*, *nrfA*, and *nirB* promoters). It is possible that NarL is competent for transcription activation only when bound upstream of the −41.5 region. The *napF* operon provides the only known example of a promoter activated by NarP but not NarL. In this control region, the common NarP- and NarL-binding site is at position −44.5, in the region normally occupied by Fnr. In this context, only NarP is competent to activate transcription from this −44.5 binding site. The positioning of this NarP- and NarL-binding site results in the Fnr-binding site being further upstream, making this an unusual Fnr-dependent promoter.

The major anaerobic in vivo transcription start site of the *napF* promoter places the Fnr-binding site at −64.5 (Fig. 1) (5). However, optimal activation of a synthetic Fnr-dependent promoter with the Fnr-binding site upstream of position −41.5 occurs when the Fnr-binding site is at position −61.5 or −71.5 (30). In this context, placing the binding site at −62.5 or −65.5 severely impaired Fnr-dependent activation (30). The Fnr-binding site of the *napF* control region, at −64.5, is therefore in a position that would not be predicted to support significant transcription activation. It is possible that the binding of NarP at position −44.5 allows Fnr to activate efficiently from this nonpermissive position. Indeed, Richet (23) noted a similarity between the *napF* promoter and the *malE* promoter. For *malE*, the Crp-binding site is at a nonpermissive position (−76.5) that is separated from the promoter by binding sites for a second activator (MalT). As Richet commented, it will be interesting to see if there is a common mechanism in the activation of the *napF* and *malE* promoters. Despite their similarities, it should be noted that *malE* regulation is more complex than *napF* regulation since elements involved in expression of the divergent *malK* promoter also play a role in *malE* expression (23).

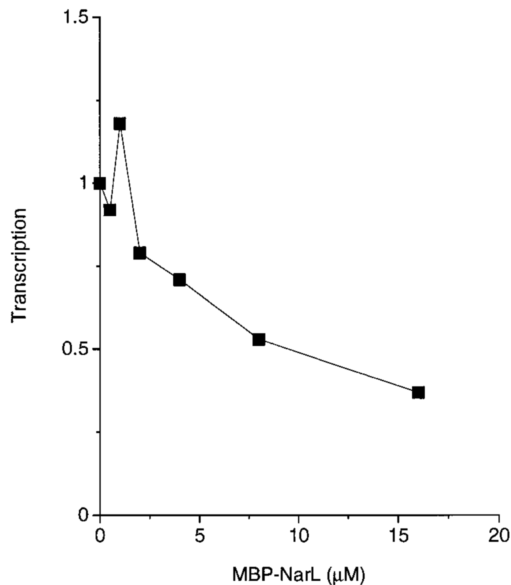


FIG. 5. Antagonization of MBP-NarP- and Fnr (D154A)-dependent *napF* transcription by MBP-NarL. Each in vitro transcription reaction mixture contained 50 nM RNA polymerase, 0.5 μM Fnr (D154A), and approximately 5 μM phosphorylated MBP-NarP. Increasing concentrations of phosphorylated MBP-NarL were added, and the change in *napF* transcription was measured as described in Materials and Methods. The amount of *napF* transcript with RNA polymerase, Fnr (D154A), and phosphorylated MBP-NarP only (no phosphorylated MBP-NarL) was arbitrarily assigned the value of 1.0.

Do Fnr and NarP activate *napF* transcription synergistically? One possible explanation for the observation that both Fnr and NarP are required for maximum *napF* transcription is that the Fnr and NarP proteins may activate transcription synergistically. By analogy with other studies of synergistic activation, Fnr and NarP may make independent contacts with RNA polymerase (4, 16, 17). Each of these contacts may have a stimulatory effect on transcription, resulting in greater transcriptional activation by Fnr and NarP together than the sum of their individual effects. An alternative hypothesis is that the binding of NarP simply allows Fnr to be an efficient activator from the −64.5 binding site, as discussed above, perhaps by bending the DNA. In this case the NarP protein may not make a stimulatory contact with RNA polymerase.

Antagonism of NarP-dependent activation by NarL. The NarL protein does not significantly activate *napF* operon expression in the presence of Fnr, whereas the NarP protein does. The explanation for this is dependent on the mechanism by which NarP activates *napF* transcription, as discussed above. NarL may be unable to make the required contact with RNA polymerase when bound to the common NarP- and NarL-binding site centered at position −44.5. Alternatively, the binding of NarL to the −44.5 site may not have the required effect on the DNA structure to enable Fnr to be an efficient activator from the −64.5 binding site. Support for the latter idea comes from the observation that DNase I footprints of the *napF* promoter with MBP-NarL or MBP-NarP are dissimilar. Both proteins protect the −44.5 region from DNase I attack, but they cause different sites to become hypersensitive to cleavage (9). One interpretation of this is that the two proteins may bend the DNA in different ways (9). Regardless of the mechanism, the NarL protein is unable to activate the *napF* promoter when bound to the −44.5 site and can be thought of as a natural positive-control mutant (relative to NarP function) in this context.

Significance of the *napF* operon transcription start site. It appeared that the in vitro *napF* transcription start site in the presence of either RNA polymerase alone or Fnr (D154A) was approximately three nucleotides upstream relative to the site in the presence of MBP-NarL or MBP-NarP (Fig. 3 and 4). We were unable to confirm this observation in vivo since a primer extension product for the *napF* transcript from a *narL narP* double-null strain was undetectable. However, we did confirm the start site of the Fnr-plus-NarP-induced promoter (+1 in Fig. 1; data not shown). In a previous study two major transcription start sites, separated by 3 or 4 nucleotides, were detected for the *napF* promoter in vivo (5). The upstream start site (−3 in Fig. 1) was detected from aerobically grown cells, and the downstream start site (+1 in Fig. 1) was detected from anaerobically grown cells. This led to the suggestion that the upstream start site was Fnr independent whereas the downstream start site was Fnr dependent. However, it is possible that the shift in the transcript start site was caused by NarL or NarP rather than by Fnr, since only the anaerobic culture may have contained nitrate or since the level of expression of the *narL* and *narP* genes during aerobic growth with nitrate is significantly lower than that during anaerobic growth with nitrate (7). It is interesting to note that the upstream transcription start site (position −3 in Fig. 1) would place the Fnr-binding site at the permissive −61.5 position (30). Furthermore, there are alternative −10 sequences at the *napF* promoter, overlapping by 3 bp (TAATATCTT; Fig. 1). Understanding *napF* operon transcription start site selection awaits further experimental tests.

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